SELECTIVE COX-2 INHIBITION FROM NON-EDIBLE PLANT EXTRACTS

Field of the Invention

The current invention is generally directed toward nutraceuticals that are nonsteroidal anti-inflammatory agents capable of inhibiting cyclooxygenase-2 (COX-2). The present invention relates to a method for inhibition of COX-2, or selective inhibition of COX-2 in an organism by administering to the organism organic extracts isolated from non-edible plants wherein such extracts inhibit COX-2 activity. The present invention also relates to purified compositions of the plant organic extracts. In addition, the current invention is directed toward a method for treating and/or preventing COX-2 mediated inflammation or inflammation-associated disorders in an organism.

Background of the Invention

The prostaglandins are a potent class of biologically active lipid derivatives that play a crucial role in the inflammatory response. The inflammatory response is a localized tissue response to injury or other trauma characterized by pain, heat, redness and swelling. Prostaglandins mediate this response by inhibiting platelet aggregation, increasing vascular permeability, increasing vascular dilation, inducing smooth-muscle contraction and causing the induction of neutrophil chemotaxis . Because of their central role in mediating the inflammatory response, significant efforts have been directed toward elucidating compositions that are capable of inhibiting the biosynthesis of prostaglandins.

Toward that end, prostaglandin biosynthesis has been extensively characterized. Prostaglandins are a group of oxygenated fatty acids that are generally derived from arachidonic acid. The biosynthesis of prostaglandins from arachidonic acid occurs in a three step process that

includes 1) hydrolysis of arachidonic acid from phospholipid precursors catalyzed by a phospholipase A_2; 2) cyclooxygenase ("COX") catalyzed oxygenation of arachidonic acid to prostaglandin G2 ("PGG2"). This COX catalyzed reaction is the first committed and rate limiting step in prostaglandin synthesis; and 3) conversion of prostaglandin G2 to the biologically active end product, prostaglandin, catalyzed by a series of synthases and reductases. Upon their synthesis, prostaglandins exit the cell and act in a hormone-like manner by effecting the target cell via G protein linked membrane receptors.

Inactivation of the COX enzyme is a natural target as a means to inhibit prostaglandin production due to this enzyme's pivotal role in the prostaglandin biosynthetic pathway. It is now known that two gene products possessing COX enzyme activity are expressed, termed COX-1 and COX-2. COX-1 was the first discovered isoform and is constitutively expressed in most tissue types. Because it is constitutively expressed, COX-1 is available to participate in activities requiring a rapid physiological response and causes the production of prostaglandins involved in "house-keeping" functions. For example, COX-1 is responsible for acute production of prostaglandins that regulate vascular homeostasis, maintain gastrointestinal integrity, and maintain kidney function. Thus, COX-1 activity is responsible for the synthesis of prostaglandins required for the maintenance of several cell types.

COX-2, on the other hand, is a recently discovered isoform that is inducibly expressed in response to numerous stimuli such as bacterial lipopolysaccharides, growth factors, cytokines, and phorbol esters. In addition, COX-2 is only expressed in a limited number of cell types including monocytes, macrophages, neutrophils, fibroblasts and endothelial cells. COX-2 expression, but not COX-1 expression, has been shown to increase in rheumatoid synovial tissue. Contrastingly, COX-2 expression is

inhibited in response to glucocorticoids and by anti-inflammatory cytokines. Thus, based upon these observations, COX-2 has been shown to be the isoform responsible for mediating the production of prostaglandins that participate in the inflammatory response and inflammatory related disorders. In addition, COX-2 has also been shown to participate in certain cancers, Alzheimer's disease, atherosclerosis, and central nervous system damage resulting from stroke, ischemia and trauma.

Corticosteroids provide one means to reduce effects associated with the inflammatory response. These potent anti-inflammatory agents exert their effect by causing a reduction in the number and activity of immune system cells via various mechanisms. However, prolonged administration of corticosteroids results in drastic side effects that limit the therapeutic value of this class of anti-inflammatory agent.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are also utilized as a means to reduce effects associated with the inflammatory response. The principal pharmaceutical effects of NSAIDs are due to their ability to prevent COX activity resulting in the inhibition of prostaglandin synthesis. Inhibition of prostaglandin synthesis by NSAIDs is anti-pyretic, analgesic, anti-inflammatory, and anti-thrombogenic. However, administration of NSAIDs may also result in severe side effects such as gastrointestinal bleeding, ulcers and incidence of renal problems. NSAIDs also inhibit both COX isoforms to varying degrees. For example, the most common NSAID, aspirin (acetylated derivative of salicylic acid), inhibits prostaglandin biosynthesis by irreversibly inactivating both COX-1 and COX-2 via acetylation of a serine residue located in the arachidonic binding domain. While aspirin inactivates both isoforms, it is 10 to 100 times more effective inactivating COX-1 as opposed to COX-2.

The selective inhibition of COX-2 has been shown to be

anti-inflammatory and analgesic without the associated gastric and kidney related toxicity problems. This phenomenon is due to the discovery of NSAIDs that are capable of inhibiting COX-2, which is responsible for the production of prostaglandins that mediate the inflammatory response, without causing the inhibition of COX-1, which is responsible for the production of prostaglandins that maintain both gastrointestinal integrity, and kidney function. Thus, the beneficial effects of NSAIDs are separable from their drastic side effects by the development of COX-2 selective inhibitors.

Toward that end, several drugs that are COX-2 selective inhibitors of prostaglandin synthesis have been developed. The most extensively characterized class of COX-2 selective inhibitor is diarylheterocycles, which include the recently approved drugs celecoxib and rofecoxib. However, other classes include, but are not limited to, acidic sulfonamides, indomethacin analogs, zomepirac analogs, chromene analogs, and di-*t*-butylphenols. For example, U.S. Pat. No. 5,380,738 describes oxazoles which selectively inhibit COX-2, U.S. Pat. No. 5,344,991 describes cyclopentenes which selectively inhibit COX-2, U.S. Pat. No. 5,393,790 describes spiro compounds which selectively inhibit COX-2, WO94/15932 describes thiophene and furan derivatives which selectively inhibit COX-2, and WO95/15316 describes pyrazolyl sulfonamide derivatives which selectively inhibit COX-2.

In order to afford an alternative to drug-based selective COX-2 therapy, it would be highly beneficial to provide nutraceuticals that inhibit COX-2, or even more preferably that selectively inhibit COX-2. A nutraceutical, in this context, is a composition that is a naturally occurring product that can safely be consumed and that exhibits COX-2 inhibitory activity. In particular, it would be highly beneficial to obtain the nutraceutical composition or extract from a plant source due to the ability to derive

a large quantity of the nutraceutical from a plant at a relatively affordable cost. These nutraceutical compositions could be utilized in the diet in a preventative manner to maintain a "healthy" physiological state. The nutraceutical compositions could also be used as a means to treat, cure or mitigate an existing inflammatory-related ailment either alone or in combination with another compound as a part of combination therapy.

Summary of the Invention

Among the several aspects of the invention therefore, is provided a method for inhibiting the activity of COX-2 in an organism, the method comprising the step of administering to the organism a therapeutically or prophylactically effective amount of an organic extract of a non- edible plant, wherein the plant is selected from the order consisting of Arales, Asterales, Coniferales, Equisetales, Euphorbiales, Geraniales, Lamiales, Lillales, Pteridophyta, Ranales, Rhamnales, Rutales, Scrophulariales, Umbellales, and Urticales.

Another aspect of the invention is a method for inhibiting the activity of COX-2 in an organism, the method comprising the step of administering to the organism a therapeutically or prophylactically effective amount of an organic extract of a plant, wherein the plant is selected from the order consisting of Arales, Asterales, Coniferales, Equisetales, Euphorbiales, Geraniales, Lamiales, Lillales, Pteridophyta, Ranales, Rhamnales, Rutales, Scrophulariales, Umbellales, and Urticales, wherein the organic extract is a purified composition obtained by a method comprising contacting the plant with an organic solvent to remove an extract from the plant wherein the extract inhibits COX-2 activity and then isolating the extract with COX-2 inhibitory activity.

Still another aspect provides a method of treating or preventing COX-2 mediated inflammation or an inflammation-

associated disorder in an organism, the method comprising administering to the organism a therapeutically or prophylactically effective amount of the purified composition of an organic plant extract wherein the purified composition is obtained by a method comprising contacting the plant with an organic solvent to remove an extract from the plant wherein the extract inhibits COX-2 activity and then isolating the extract with COX-2 inhibitory activity.

Other features of the present invention will be in part apparent to those skilled in the art and in part pointed out in the detailed description provided below.

<u>Brief Description of the Drawings</u>

These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims and accompanying figures where:

Figure 1 depicts COX-2 > COX-1 inhibition by an extract isolated from *Arisaema heterophyllum*.

Figure 2 depicts COX-2 > COX-1 inhibition by an extract isolated from *Mohonia fortunei*.

Figure 3 depicts COX-2 > COX-1 inhibition by an extract isolated from *Hydrastis canadensis*.

<u>Abbreviations and Definitions</u>

To facilitate understanding of the invention, a number of terms and abbreviations as used herein are defined below:

"Purified" means partially purified and/or completely purified. Thus, a "purified composition" may be either partially purified or completely purified.

"Extract" means crude extract, purified extract, and purified composition obtained by purification of the extract.

"COX activity" means the ability of either COX isoform, COX-1 or COX-2, to catalyze the oxygenation reaction of

arachidonic acid to PGG2.

"COX inhibitor or COX inhibition" means a composition, agent or extract, purified or otherwise, that prevents either COX isoform, COX-1 or COX-2, from catalyzing the oxygenation reaction of arachidonic acid to PGG2 either in whole or in part.

"Selective inhibition of COX-2" means a composition, agent, or extract, purified or otherwise, which selectively inhibits COX-2 activity over COX-1 activity as determined by the ratio of the percentage of COX-2 inhibition divided by the percentage of COX-1 inhibition, unless otherwise indicated herein.

"IC_{50}" means the concentration (in mol L^{-1}) that reduces a specified response to 50% of its former value. As used herein this value measures the amount of composition, agent or extract (ug extract/ml solvent) causing 50% inhibition of PGE2 production. The IC_{50} value may be used to determine COX-2 selectivity as specifically set-forth herein.

"Plant or parts thereof" means either the whole plant, or any part of the plant such as an aerial part, fruit, leaf, stem, or root and any combination thereof.

"Order", as utilized herein, is a taxonomic category of related organisms with a category consisting of a number of similar families.

"Family", as utilized herein, is a taxonomic category of related organisms ranking below the order and above the genus.

"Species", as utilized herein, is a fundamental taxonomic category ranking below a genus and consisting of a group of closely related individuals.

COX = the enzyme cyclooxygenase

COX-1 = the isoform cyclooxygenase-1

COX-2 = the isoform cyclooxygenase-2

NSAIDs = nonsteroidal anti-inflammatory drugs

PGE2 = prostaglandin E2

Description of the Preferred Embodiment

Applicants have discovered that organic extracts of certain non-edible plants or parts therefrom inhibit COX-2 activity. Applicants have also discovered that organic extracts of certain non-edible plants or parts therefrom selectively inhibit COX-2 activity. The inhibitory effect is selective because inhibition of COX-2 is greater than inhibition of COX-1. Consequently, organic extracts of such plants or parts therefrom may be used to selectively inhibit the activity of COX-2 in an organism without causing an equivalent inhibition of COX-1 activity. Advantageously, these organic extracts are nutraceuticals that may be safely consumed and provide an alternative to traditional drug-based therapy for COX-2 inhibition.

Accordingly, the extracts of the present invention preferably inhibit COX-2 activity more than COX-1 activity. Preferably, the inhibitory effect of the plant extract on COX-2 is at least about two times greater than its inhibitory effect on COX-1. More preferably, the inhibitory effect on COX-2 is at least about 10 times greater than the inhibitory effect on COX-1. COX enzyme inhibition and selectivity may be determined in accordance with any method generally known to those of ordinary skill in the field, as set forth in more detail below.

In addition to inhibiting COX-2, the organic extracts of the present invention are preferably isolated from a non-edible plant. In general, plants are classified as non-edible if they are utilized for a purpose other than nourishment. For example, medicinal plants are considered non-edible because they are consumed for the purpose of correcting symptoms of illness and are considered too potent to be consumed on a daily basis. Classification of plants as edible versus non-edible may be accomplished utilizing references commonly known to those skilled in the art for example, such references include, NAPRALERT; Tyozaburo Tanaka, (Edited by Sasuke Nakoa) Tanaka's Cyclopedia of

Edible Plants of the World, Keigaku Publishing Co., Tokyo, Japan, 1976; Stephen Facciola, Cornucopia II: A Source Book of Edible Plants, Kampong Publications, Vista, California, 1998; James A. Duke, Database of Phytochemical constituents of GRAS Herbs and Other Economic Plants, CRC Press, Boca Raton, Florida, 1992; and George Macdonald Hocking, Dictionary of Natural Products, Plexus Publishing, Inc., Medford, New Jersey, 1997. The contents of these references are hereby incorporated in their entirety.

In a particularly preferred embodiment, organic extracts are isolated from non-edible plants of the following plant orders: Arales, Asterales, Coniferales, Equisetales, Euphorbiales, Geraniales, Lamiales, Lillales, Pteridophyta, Ranales, Rhamnales, Rutales, Scrophulariales, Umbellales, and Urticales. The ability of extracts isolated from non-edible plants of these particular orders to inhibit COX-2, selectively inhibit COX-2 and their use as non-edible plants are set-forth below in **Tables 1-5 and Figures 1-3.**

In order to prepare the extracts of the invention, a non-edible plant or parts thereof is ground into a fine powder, the resultant powder is extracted with a solvent, and the extraction solvent is removed from the extract. The whole plant may be used or parts of the plant including an aerial part, fruit, leaf, stem, or root and any combination thereof may be used. If desired, the resultant extract may be further purified to yield a purified extract or one or more purified compositions. The grinding step may be accomplished by any commonly known method for grinding a plant substance. For example, the plant or parts thereof may be passed through a grinder to obtain a fine powder.

After the plant or parts thereof have been ground into a fine powder, they are combined with an extraction solvent. The solution is then stirred at a temperature, and for a period of time, that is effective to obtain an extract with the desired inhibitory effects on the activity of COX-2. The solution is preferably not overheated, as this may

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result in degradation and/or denaturation of proteins in the extract. The solution may be stirred at a temperature between about room temperature (25° C) and the boiling point of the extraction solvent. Preferably, the solution is stirred at about room temperature.

The length of time during which the plant powder is exposed to the extraction solvent is not critical. Up to a point, the longer the plant powder is exposed to the extraction solvent, the greater is the amount of extract that may be recovered. Preferably, the solution is stirred for at least 1 minute, more preferably for at least 15 minutes, and most preferably for at least 60 minutes.

The extraction process of the present invention is desirably carried out using an organic solvent or a mixture of organic solvents. Organic solvents which may be used in the extraction process of the present invention, include but are not limited to hydrocarbon solvents, ether solvents, chlorinated solvents, acetone, ethyl acetate, butanol, ethanol, methanol, isopropyl alcohol and mixtures thereof. Hydrocarbon solvents which may be used in the present invention include heptane, hexane and pentane. Ether solvents which may be used in the present invention include diethyl ether. Chlorinated solvents which may be used in the present invention include dichloromethane and chloroform. Preferably, the solvent utilized for such extraction is a nonpolar organic solvent, such as dichloromethane or hexane.

The relative amount of solvent used in the extraction process may vary considerably, depending upon the particular solvent employed. Typically, for each 100 grams of plant powder to be extracted, about 500 ml of extraction solvent would be used. The organic solvent may be removed from the extract by any method known in the field of chemistry for removing organic solvents from a desired product, including, for example, rotary evaporation.

It is believed that the inhibitory effect of the plant

extract of this invention on the activity of COX-2 is due to the presence of one or more compounds in the extract. Compounds present in the extract which inhibit the activity of COX-2 may be isolated and purified by those of ordinary skill in the art employing methods known in the art. For example, column chromatography and fractional distillation may be used to obtain pure compounds from the plant extract of this invention.

The isolation and purification of particular compounds from the organic plant extracts of this invention may be performed as described in Resch, et al., J. Nat. Prod., 61, 347-350 (1998), the entire contents of which are incorporated by reference herein. The methods disclosed therein may be used to isolate and purify compositions which inhibit COX-2.

The ability of a particular organic extract to inhibit COX-1 or COX-2 is preferably determined by performing COX activity assays utilizing recombinant COX-1 and COX-2. The COX-1 and COX-2 genes may be subcloned from a variety of organisms, however in a preferred embodiment such genes are isolated from human or murine sources, using a variety of procedures known to those skilled in the art and detailed in, for example, Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, (1989) and Ausabel et al., Short Protocols in Molecular Biology, 3rd. ed., John Wiley & Sons (1995). Additionally, the subcloned portion of the particular COX gene may be inserted into a vector by a variety of methods. In a preferred method, the sequence is inserted into an appropriate restriction endonuclease site(s) in a baculovirus transfer vector pVL1393 utilizing procedures known to those skilled in the art and detailed in, for example, Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, (1989) and Ausubel et al., *Short Protocols in Molecular Biology*, 3rd ed., John Wiley & Sons (1995).

The recombinant baculoviruses may be isolated by transfecting an appropriate amount of baculovirus transfer vector DNA into a sufficient quantity of SF9 insect cells along with linearized baculovirus plasmid DNA by the calcium phosphate method or any other method generally know to those skilled in the art. (<u>See</u> M.D. Summers and G.E. Smith, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agric. Exp. Station Bull. 1555 (1987)). Recombinant viruses may be purified by three rounds of plaque purification and high titer (10^7-10^8 pfu/ml) stocks of virus may be prepared.

Preferably, for large scale production, cells may be infected in approximately 10 liter fermentors (0.5×10^6/ml) with the recombinant virus stock such that the multiplicity of infection is greater than about 0.1. After several hours the cells are centrifuged and the cell pellet is homogenized in an appropriate buffer such as Tris/sucrose (50 mM/25%, pH 8.0). The homogenate may then be centrifuged at an appropriate speed and for an appropriate time (such as 10,000 × G for 30 minutes) so as to cause the homogenate to separate into a pellet and supernatant fraction. The resultant supernatant fraction will contain the desired product and may be stored at -80° C until use.

In order to test organic extracts for COX-2 inhibition and selectivity, standard COX-1 and COX-2 assays may be performed by employing ELISA procedures generally known to those skilled in the art. In such procedures, COX-1 and COX-2 activities are assayed as PGE_2 formed/mg protein/time using ELISA to detect the amount of PGE_2 synthesized from arachindonic acid. PGE_2 formation may be measured using PGE_2 specific antibody. Indomethacin, a non-selective COX-2/COX-1 inhibitor, may be employed as a positive control. The relative ability of various organic extracts to inhibit COX-1 or COX-2 at a particular concentration may be determined by comparing the IC_{50} value expressed as mg extract/ml solvent resulting in a 50% inhibition of PGE2 production.

Selective inhibition of COX-2 may then be determined by the IC_{50} ratio of COX-1/COX-2. Additionally, any other means to determine COX inhibition known to those generally skilled in the art may be employed.

The extracts of this invention may be used to manage, prevent and/or treat an organism having, or at risk for developing, a condition which is mediated in whole or in part by COX-2. Accordingly, conditions which may be benefited by inhibition of COX-2 or selective inhibition of COX-2 include, but are not limited to, the treatment of inflammation in an organism, and for treatment of other inflammation-associated disorders, such as, an analgesic in the treatment of pain and headaches, or as an antipyretic for the treatment of fever. For example, extracts of the invention would be useful to treat arthritis, including but not limited to rheumatoid arthritis, spondyloarthopathies, gouty arthritis, osteoarthritis, systemic lupus erythematosus and juvenile arthritis. Such extracts of the invention would be useful in the treatment of asthma, bronchitis, menstrual cramps, tendinitis, bursitis, skin-related conditions such as psoriasis, eczema, burns and dermatitis, and from post-operative inflammation including ophthalmic surgery such as cataract surgery and refractive surgery. Extracts of the invention also would be useful to treat gastrointestinal conditions such as inflammatory bowel disease, Crohn's disease, gastritis, irritable bowel syndrome and ulcerative colitis, and treatment of cancer, including but not limited to the following types of cancer: colon, breast, prostate, bladder, or lung. In yet another preferred use, the extracts of the present invention may also be utilized as chemopreventive agents. Extracts of the invention would be useful in treating inflammation in such diseases as vascular diseases, migraine headaches, periarteritis nodosa, thyroiditis, aplastic anemia, Hodgkin's disease, sclerodoma, rheumatic fever, type I diabetes, neuromuscular junction disease including

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myasthenia gravis, white matter disease including multiple sclerosis, sarcoidosis, nephrotic syndrome, Behcet's syndrome, polymyositis, gingivitis, nephritis, hypersensitivity, swelling occurring after injury, myocardial ischemia, and the like. The extracts would also be useful in the treatment of ophthalmic diseases, such as retinitis, retinopathies, uveitis, ocular photophobia, and of acute injury to the eye tissue. The extracts would also be useful in the treatment of pulmonary inflammation, such as that associated with viral infections and cystic fibrosis. Additionally, the extracts would be beneficial for the treatment of certain central nervous system disorders such as cortical dementias including Alzheimer's disease. The extracts of the invention are useful as anti-inflammatory agents, such as for the treatment of arthritis, with the additional benefit of having significantly less harmful side effects. These extracts would also be beneficial in the treatment of allergic rhinitis, respiratory distress syndrome, endotoxin shock syndrome, atherosclerosis and central nervous system damage resulting from stroke, ischemia and trauma. Additionally, the extracts would be useful in the treatment of pain, including but not limited to postoperative pain, dental pain, muscular pain, and pain resulting from cancer.

The present extracts may also be employed either alone or in combination with other compounds as a part of combination therapy, partially or completely, in place of other conventional anti-inflammatory agents. For example, such as together with steroids, NSAIDs, 5-lipoxygenase inhibitors, leukotriene receptor antagonists, LTA4 hydrolase inhibitors, and LTC4 synthase inhibitors. Preferably, with combination therapy one will typically combine a drug or drugs and a nutraceutical, such as a plant extract of the current invention, in a manner such that the drug and the nutraceutical have different mechanisms of action, but yet target the same disease. For example, in a typical

selection of agents for use in combination therapy to treat arthritis, one could utilize a plant extract of the present invention, which exhibits selective COX-2 inhibition with another agent known to attenuate inflammation associated with arthritis via an independent mechanism.

Those of ordinary skill in the art of preparing pharmaceutical formulations can readily formulate pharmaceutical compositions having plant extracts using known excipients (e.g., saline, glucose, starch, etc.). Similarly, those of ordinary skill in the art of preparing nutritional formulations can readily formulate nutritional compositions having plant extracts. And those of ordinary skill in the art of preparing food or food ingredient formulations can readily formulate food compositions or food ingredient compositions having plant extracts.

In addition, those of ordinary skill in the art can readily determine appropriate dosages that are necessary to achieve the desired therapeutic or prophylactic effect upon oral, parenteral, rectal and other administration forms. Typically, *in vivo* models (i.e., laboratory mammals) are used to determine the appropriate plasma concentrations necessary to achieve a desired mitigation of inflammation related conditions.

The extracts of the present invention may be employed for the treatment and/or prevention of inflammation-related disorders, as identified above, in a number of organisms. Besides being useful for human treatment, these extracts are also useful for veterinary treatment of companion animals, exotic animals and farm animals, including mammals, rodents, avians, and the like. More preferred animals include horses, dogs, cats, sheep, and pigs.

The detailed description set-forth above is provided to aid those skilled in the art in practicing the present invention. Even so, this detailed description should not be construed to unduly limit the present invention as modifications and variation in the embodiments discussed

herein can be made by those of ordinary skill in the art without departing from the spirit or scope of the present inventive discovery.

All publications, patents, patent applications and other references cited in this application are herein incorporated by reference in their entirety as if each individual publication, patent, patent application or other reference were specifically and individually indicated to be incorporated by reference.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

<u>Examples</u>

Sample Preparation

Plants or parts thereof were dried and sliced ("sample"). Samples of organic extracts were prepared from the non-edible plants listed in **Table 1**. The plant order and families that the various samples were prepared from are also set-forth in **Table 1**. In addition, details regarding the use of these some of these plants as medicinals is set-forth in **Table 2**. The particular sample was then ground into a fine powder using a coffee grinder. Approximately 100 grams of the resulting powder were added to approximately 500 ml of dichloromethane and stirred at room temperature for about 1 hour. The solvent was then removed by rotary evaporation, leaving several grams of the particular extract.

Inhibitory Effect of Various Plant Organic Extracts on COX-1 and COX-2 Activity

The particular extracts resulting from the sample preparation procedure detailed above were each evaluated for

inhibition of COX-1 and COX-2. The COX-1 and COX-2 inhibition activities were determined in *vitro* according to the method of Gierse et al., *J. Biochem.*, 305, 479-484 (1995). This method is summarized below.

<u>Preparation of recombinant COX baculoviruses</u>

Recombinant COX-1 was prepared by cloning a 2.0 kb fragment containing the coding region of human or murine COX-1 into a BamH1 site of the baculovirus transfer vector pVL1393 (Invitrogen) to generate the baculovirus transfer vectors for COX-1 according to the method of D.R. O'Reilly et al., *Baculovirus Expression Vectors: A Laboratory Manual* (1992).

Recombinant baculoviruses were then isolated by transfecting 4 mg of baculovirus transfer vector DNA into (2 $\times 10^8$) SF9 insect cells along with 200 mg of linearized baculovirus plasmid DNA by the calcium phosphate method. (<u>See</u> M.D. Summers and G.E. Smith, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agric. Exp. Station Bull. 1555 (1987)). Recombinant viruses were purified by three rounds of plaque purification and high titer (10^7-10^8 pfu/ml) stocks of virus were prepared.

For large scale production, SF9 insect cells were infected in 10 liter fermentors (0.5×10^6/ml) with the recombinant baculovirus stock such that the multiplicity of infection was 0.1. After 72 hours the cells were centrifuged and the cell pellet was homogenized in Tris/sucrose (50 mM/25%, pH 8.0) containing 1% of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The homogenate was then centrifuged at 10,000 × G for 30 minutes, and the resultant supernatant was stored at -80° C until use.

Recombinant COX-2 was prepared by cloning a 2.0 kb fragment containing the coding region of human or murine

COX-2 in accordance with the same method described above for COX-1.

Assay for COX-1 and COX-2 Activities

COX-1 and COX-2 activities were assayed as prostaglandin E2 (PGE2) formed/mg protein/time using ELISA to detect PGE2 synthesized from arachindonic acid. CHAPS-solubilized insect cell membranes containing recombinant COX-1 or COX-2 enzyme were incubated in a potassium phosphate buffer (50 mM, pH 8.0) containing epinephrine, phenol, and heme. Compounds or were pre-incubated with the appropriate enzyme or plant extract for approximately 10-20 minutes. Arachidonic acid (10 mM) was then added to the mixture and the reaction was permitted to occur for ten minutes at room temperature (25° C).

Any reaction between the arachidonic acid and the enzyme was stopped after ten minutes by transferring 40 ml of reaction mixture into 160 ml ELISA buffer and 25 mM indomethacin. Indomethacin, a non-selective COX-2/COX-1 inhibitor, was utilized as a positive control. The PGE2 formed was measured by standard ELISA technology utilizing a PGE2 specific antibody (Cayman Chemical).

Approximately 200 mg of each extract obtained from the sample preparation procedure set-forth above were each individually dissolved in 2 ml of dimethyl sulfoxide (DMSO) for bioassay testing to determine the COX-1 and COX-2 inhibitory effects of each particular extract. Potency was determined by the IC_{50} value expressed as mg extract/ml solvent resulting in a 50% inhibition of PGE2 production. Selective inhibition of COX-2 was determined by the IC_{50} ratio of COX-1/COX-2. The results of these bioassays performed utilizing extract isolated from the plant family indicated are reported in **Tables 1-5** **and Figures 1-3** delineated below.

Table 1 below sets forth results of screening extracts of plants isolated from the orders, families, genera, and

species indicated. A primary screen (indicated as 1° assay in Table 1) was performed in order to identify particular extracts that inhibited COX-2 at a concentration of 10 ug/ml. The extracts were then subjected to a confirmation screen to determine the extent of COX-2 inhibition at three different concentrations (10 ug/ml, 3.3 ug/ml and 1.1 ug/ml). The extracts were then tested for their ability to inhibit COX-1 at a concentration of 10 ug/ml. The percentage of COX inhibition is indicated as a percentage in each column, with a higher percentage indicating a greater degree of COX inhibition. In addition, the IC_{50} value for COX-1 and COX-2 was also determined for certain extracts as indicated in Table 1. The selectivity for these extracts was then determined by the IC_{50} ratio of COX-1/COX-2, as set-forth above. The COX-2 selectivity of extracts whose IC_{50} value was not determined may be calculated by dividing the percentage of COX-2 inhibition (at a concentration of 10 ug/ml) by the percentage of COX-1 inhibition (at a concentration of 10 ug/ml).

Table 1: Extracts from Non-Edible Plants that Inhibit COX-2

Order	Family	Genus	Species	Common name	Part	1° assay COX-2 (% inhib.) 10 ug/ml	Confirmation assay COX-2 (% inhib.) 10 ug/ml	3.3 ug/ml	1.1 ug/ml	COX-1 (% inhib.) 10 ug/ml	IC50 (ug/ml) COX-2	IC50 (ug/ml) COX-1	Selectivity COX-2/COX-1
Arales[9]	Araceae	Arisaema	heterophyllum			79%	87%	**	0.2	-46%	2	25	12.5
Asterales	Asteraceae	Artemisia	anomola	herba artemisiae	PL	76%	71%	30%	27%	15%	***	***	***
Asterales	Asteraceae	Microglossa	angolensis			86%	66%	**	27%	-100%	***	***	***
Asterales	Asteraceae	Senecio	discifolius	ragwort		75%	76%	41%	32%	18%	***	***	***
Asterales	Asteraceae	Sigesbeckia	orientalis	St. Paul's wort		84%	67%	47%	20%	14%	***	***	***
Asterales	Asteraceae	Spilanthes	mauritiana	isisilili (Africa)		83%	81%	63%	26%	37%	***	***	***
Coniferales	Cupressaceae	Biota	orientalis		SD	78%	79%	67%	73%	62%	***	***	***
Equisetales	Equisetaceae	Equisetum	hyemale		ST	75%	88%	**	30%	-32%	***	***	***
Euphorbiales	Euphorbiaceae	Acalypha	ornata			78%	40%	19%	21%	-6%	***	***	***
Euphorbiales	Euphorbiaceae	Phyllanthus	guineensis	Guinea spurge		81%	75%	39%	37%	26%	***	***	***
Geraniales	Geraniaceae	Geranium	carolinianum	Carolina geranium		79%	100%	**	22%	35%	***	***	***
Lamiales	Lamiaceae[4]	Salvia	miltorrhiza	radix salviae miltorrhizae; sage	RT	86%	93%	**	49%	27%	***	***	***
Lamiales	Lamiaceae[4]	Solenostemon	latifolius	inuka; nidayana		79%	68%	40%	27%	12%	***	***	***
Liliales	Liliaceae	Paris	chinensis	rhizome of Chinese paris	RT	75%	71%	40%	12%	25%	***	***	***
Pteridophyta	Polypodiaceae[11]	Platycerium	elephantotis			93%	61%	31%	34%	4%	***	***	***
Ranales	Berberidacea	Mahonia	fortunei	shi da gong lao ye (China)		81%	100%	**	25%	11%	0.8	7	8.8
Ranales	Ranunculaceae	Clematis	hirsuta	bisinda (Africa)		80%	57%	**	25%	17%	***	***	***
Ranales	Ranunculaceae	Hydrastis	canadensis	golden seal		86%	93%	**	25%	22%	3.5	15	4.3
Ranales	Ranunculaceae	Ranunculus	multifidus	wild buttercup		89%	98%	**	35%	36%	***	***	***
Rhamnales	Rhamnaceae	Rhamnus	purshiana	cascara sagrada	BK	80%	95%	**	74%	69%	***	***	***
Rhamnales	Vitaceae	Cyphostemma	adenocaule	iwengere		82%	58%	43%	16%	11%	***	***	***
Rutales[1]	Rutaceae	Zanthoxylum	americanum	prickly ash		79%	79%	47%	20%	60%	***	***	***
Scrophulariales	Acanthaceae	Justicia	secunda	Saint John's bush		76%	80%	**	18%	-58%	***	***	***
Umbellales	Apiaceae[5]	Angelica	dahurica	pai-chi	RT	76%	71%	38%	20%	39%	***	***	***
Urticales	Urticaceae	Fleurya	ovalifolia	stinging nettle		83%	47%	26%	23%	-20%	***	***	***

* Primary screen performed at three concentrations. Samples were not repeated in a COX-2 confirmation assay.
** No data due to assay error.
*** Not tested.

[1]Brassicales also classified as Sapindales or Rutales
[4]Lamiaceae also classified as Labiatae
[5]Apiaceae also classified as Umbelliferae
[9]Pandanales also classified as Arales or Alismatales
[11]Polypodiaceae also classified as Pteridophyta

The order, family, genus, and species of each plant whose extract was tested for COX-2 and COX-1 inhibitory activities are shown.

Table 2 below provides a description detailing the medicinal use of some of the plant extracts tested for COX-2 inhibition as set-forth in **Table 1**. In addition, a comprehensive listing of references known to those generally skilled in the art is provided detailing plant uses.

Table 2 - MEDICINAL USES OF PLANT EXTRACTS

Scientific Name	Common Name or Comment	Isolate/ Chemical ID	Sample ID	Extract #	Reference
Acalypha ornata	Used in scabies, leprosy	78459	914108		5
Angelica dahurica	Pai-chi	78957	914526		5
Arisaema heterophyllum	No common name available	76943	912370		5
Artemesia anomola	Herba Artemisiae	79489	914823		5
Biota orientalis	Species not found, but others medicinal	79560	914894		1
Clematis hirsuta	bisinda (Africa)	78472	914121		6
Cyphostemma adenocaule	iwengere	78480	914128		2
Equisetum hyemale	Species not found, but others are medicinal.	79032	914601		5
Fleurya ovalifolia	Stinging nettle	78460	914109		5
Geranium carolinianum	Carolina geranium	76818	912282		5
Hydrastis	golden seal	79476	914810		2

canadensis	tea				
Justicia secunda	Saint John bush	77160	912439		5
Mahonia fortunei	shi da gong lao ye (China)	76787	912259		5
Microglossa angolensis	Species not found, but other species medicinal.	78453	914102		5
Paris chinensis	Rhizome of Chinese Paris	79044	914613		1
Phyllanthus guineensis	Guinea spurge	78449	914099		5
Platycerium elephantotis	Species not found. A genus of medicinal ferns.	78461	914110		5
Ranunculus multifidus	wild buttercup	78455	914104		5
Rhamnus purshiana	cascara sagrada	79373	914707		
Salvia miltorrhiza	Radix salviae miltorrhizae	79324	914658		
Senecio discifolius	Ragwort	78479	914127		5
Sigesbeckia orientalis	St. Paul's wort	78452	914101		5
Solenostemon latifolius	inuka, nidayana	78476	914124		5
Spilanthes mauritiana	isisilili (African)	78465	914114		5
Zanthoxylum americanum	prickly ash bark	80396	922769		5

References

1. NAPRALERT (NATural Products ALERT), which currently contains the extracted information from over 116,000 scientific research articles and books from 1650 A.D. to the present. The NAPRALERT database is housed and maintained by the Program for Collaborative Research in the

Pharmaceutical Sciences (PCRPS), within the Department of Medicinal Chemistry and Pharmacognosy, in the College of Pharmacy of the University of Illinois at Chicago, 833 South Wood Street (M/C 877), Chicago, IL 60612, U.S.A.

2. Tyozaburo Tanaka, (Edited by Sasuke Nakao) *Tanaka's Cyclopedia of Edible Plants of the World*, Keigaku Publishing Co., Tokyo, Japan, 1976.

> This is a compendium of about 11,000 species of plants, including the essential wild species of the world. This book is considered to be one of the principle references on the world's edible plants.

3. Stephen Facciola, *Cornucopia II: A Source Book of Edible Plants*, Kampong Publications, Vista, California, 1998.

> This book records the more than 3,000 species available in the U.S. and abroad.

4. James A. Duke, *Database of Phytochemical Constituents of GRAS Herbs and Other Economic Plants*, CRC Press, Boca Raton, FL, 1992.

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5. George Macdonald Hocking, *Dictionary of Natural Products*, Plexus Publishing, Inc., Medford, NJ, 1997.

> "Terms in the field of Pharmacognosy relating to natural medicinal and pharmaceutical materials and the plants, animals and minerals from which they are derived." The work contains over 18,000 entries.

6. Enrique Sanchez-Monge, *Flora Agricola: Taxonomia de las Magnoliofitas (Angiospermas) de interes agricola, con excepcion de las de aprovechamiento exclusivamente ornamental o forestall*, Ministerio de Agriculture, Pesca y Alimentacion, Madrid, Spain, (date unknown).

An excellent reference work in Spanish with descriptions of plants, common names in many languages and commercial use of agricultural organisms of the world.

7. Anthony R. Torkelson, *The Cross Name Index to Medicinal Plants, Volumes ! - IV,* CRC Press, Boca Raton, FL, (1998-1999).

8. Umberto Quattrocchi, *CRC World Dictionary of Plant Names: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology* (Volumes 1-4), CRC Press, Boca Raton, FL (2000).

9. W[3]TROPICOS, a web site providing access to the Missouri Botanical Garden's VAST (VAScular Tropicos) nomenclatural database and associated authority files.

10. *Webster's Ninth New Collegiate Dictionary*, Merriam-Webster Inc., Springfield, Massachusetts, (1983).

Tables 3-5 further illustrate the ability of certain extracts isolated from the families identified in Table 1 to selectively inhibit COX-2. A total of 6 different concentrations of the various extracts were tested for their ability to inhibit both COX-1 and COX-2. The IC_{50} value for COX-1 and COX-2 was also determined and a selectivity ratio was then calculated as set forth above. **Figures 1-3** are graphs that depict the data shown in **Tables 3-5** as indicated.

Table 3 - Extract isolated from *Arisaema heterophyllum*

Amount of Extract (mg/ml)	COX-1 Activity Relative to Control	COX-2 Activity Relative to Control
100	17%	4%
33.3	42%	8%
11.1	68%	16%
3.70	90%	37%
1.23	105%	59%
0.41	129%	78%

IC_{50} (mg/ml) COX-1	IC_{50} (up/ml) COX-2	COX-2 Selectivity Ratio
25	2	12.5

Table 4 - Extract isolated from *Mahonia fortunei*

Amount of Extract (mg/ml)	COX-1 Activity Relative to Control	COX-2 Activity Relative to Control
100	10%	1%
33.3	27%	6%
11.1	41%	9%
3.70	64%	22%
1.23	85%	36%
0.41	118%	74%

IC_{50} (mg/ml) COX-1	IC_{50} (mg/ml) COX-2	COX-2 Selectivity Ratio

7	0.8	8.8

Table 5 -Extract isolated from *Hydrastis canadensis*

Amount of Extract (mg/ml)	COX-1 Activity Relative to Control	COX-2 Activity Relative to Control
100	5%	5%
33.3	24%	10%
11.1	59%	20%
3.70	70%	46%
1.23	84%	79%
0.41	90%	94%

IC_{50} (mg/ml) COX-1	IC_{50} (mg/ml) COX-2	COX-2 Selectivity Ratio
15	3.5	4.3

As illustrated by these data, the organic extracts isolated from the indicated plant families inhibit COX-2. In fact, one of the extracts selectively inhibits COX-2 over COX-1 by greater than 10 fold. In view of the above, it will be seen that the several objectives of the invention are achieved and other advantageous results attained.